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(57) Abstract

Identification of the human insulin receptor binding site sequence of certain spatial molecular structures conforming to such binding site and of certain insulinomimetic sequences including the binding site sequence are disclosed.

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INSULINOMIMETIC AND INSULIN RECEPTOR BINDING SITE PEPTIDES

RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 213,918 filed June 30, 1988 entitled "Insulin Receptor Binding Site" and also a continuation-in-part of application Serial No. 292,099 filed December 30, 1988 entitled "Insulinomimetic and Insulin Receptor Binding Site Peptides". The specifications, figures and claims of these applications are incorporated into this application by reference.

FIELD OF THE INVENTION

This invention relates to the amino acid residue sequences which bind the human insulin molecule or the insulin-like growth factor I (IGF-I) molecule and to linear peptides endowed with insulinomimetic properties which include the same or similar sequences.

BACKGROUND OF THE INVENTION

The cDNA of both the insulin and the IGF-I receptors have been cloned and sequenced. Expression in mammalian cells of biologically active receptors has been achieved. See United States patent 4,761,371; Ebina, et al., Cell 46:747-758 (1985); Ullrich, et al., Nature 313:756-761 (1985); and Ullrich, et al., EMBO J. 5:2503-2512 (1986). Ebina and Ullrich utilize different sequence numbers. The Ullrich sequence numbers are used exclusively herein.

The receptors are glycoproteins which consist of a heterotetramer of two extracellular α subunits and two transmembrane β subunits which have both an extracellular and an intracellular portion. The α subunits contain the insulin binding site. It is plausible that each α subunit may contain one binding site. The insulin receptor has homology with the IGF-I receptor and with the epidermal growth factor receptor, the human c-erb-2 oncogene, and the oncogene-encoded tyrosine kinase.

Knowledge of receptor structures is limited to the primary sequence deduced from cDNA cloning. This information has produced few clues as to the precise localization of the insulin or IGF-I binding domain of the α subunits, each of which contains more than 700 amino acid residues. Nor is definitive information concerning the secondary or tertiary structure of the receptors available.

The discovery of insulin-like peptides has long been a major challenge to the diabetes related pharmaceutical industry. Apparently, the only previous description of a linear peptide purportedly having insulin-like activity is found in publications by Weitzel, et al., Hoppe-Seyler's Z. Physiol. Chem. 352:1005-1013 (1971); ibid., 1735-1738 (1971); ibid., 357:187-200 (1976). These publications report that the terminal portion of insulin's B-chain is active. This claim was never confirmed by others. Per contra, the C-terminal portion of the B-chain (B23-30) has been used as a negative control in experiments which reveal the insulin-like peptides of this invention.

SUMMARY OF THE INVENTION

This invention entails identification of receptor domains involved in the binding of insulin or IGF-I molecules, the amino acid residue sequences of such domains, and their secondary and tertiary structure. It includes natural and synthetic fragments of such domains and sequences which are effective in implementation of the binding and recognition of the insulin or IGF-I molecule by the receptors, as well as physical and graphic representations of these domains. The use of such fragments and templates derived therefrom to design, for example, insulinomimetic drugs is an objective of the invention.

A seminal aspect of the invention is the discovery that the human insulin receptor domain is insulinomimetic per se and that it includes shorter sequences which are similarly endowed. Synthetic and purified natural amino acid residue sequences, many of which are insulinomimetic and constitute, in whole or in part, a binding site for the human insulin and IGF-I molecules, are provided.

The invention includes the discovery that the receptor domain strongly aggregates in solution with apparent capability to bind an intact insulin receptor on cells and activate it with resulting insulin-like activity, e.g., the activation of lipogenesis in isolated rat adipocytes.

Another aspect of the invention includes derivatives and modifications of such peptides, for example, shortened sequences of minimal structure required for binding or insulinomimetic action and cyclization or derivatization to impart or enhance solubility in the gastrointestinal tract.

The insulinomimetic peptides of the invention and derivatives and modifications thereof may be administered to diabetic patients in lieu of insulin, either subcutaneously, intranasally or orally. These peptides of the invention are useful in biochemical experiments in vitro and in vivo to study the mechanisms of insulin activity.

Yet another aspect of this invention comprises physical and graphical representations of the spatial or three-dimensional structure of these peptides and the use of such representations as templates for the design of insulinomimetic and other drugs.

DESCRIPTION OF THE FIGURES

Figure 1A is a computer-generated graphic model of the insulin dimer interface.

Figure 1B is a computer-generated graphic model of the insulin sequence B19-30 of one insulin monomer in the dimer interface replaced by sequence 83-94 of the insulin receptor.

Figure 2 depicts the homology of various receptor fragments with the C-terminal end of the insulin B-chain.

Figure 3 is a computer-generated secondary structure and hydropathy profile of the α subunit.

Figures 4A-4D are computer-generated graphic models from four different angles of the α subunit sequence which includes residues 83-94.

Figures 5A-5B are similarly computer-generated graphic models from two different angles of the entire domain including residues 83-94 in a β-sheet structure and residues 95-103 in an α-helix consistent with Figure 3.

Figure 6 is an insulin competition curve with synthetic receptor peptide.

Figure 7 depicts homology between C-terminal ends of insulin and IGF-I B-chain and receptors for insulin, IGF-I and EGF.

Figures 8A and 8B are graphic representations of the insulin-insulin dimer pair and of the insulin-insulin receptor domain including the residues 83-94. Figure 8C is a graphic representation of the IGF-I receptor domain-insulin pair. In each of the figures, the graphic representation of "insulin" appears on the right.

Figures 9A-D are graphic models from four different angles of the IGF-I receptor domain, including the residues 77-97.

Figure 10 depicts the insulinomimetic effect of certain receptor peptides.

Figure 11 depicts the range of concentration over which a peptide of Sequence I binds to insulin.

Figure 12 shows that the lipogenetic activity of a Sequence V peptide is inactivated by the polyclonal anti-insulin antibody Sigma #1-8510.

Figure 13 shows that the stimulation of fat cell lipogenesis by the peptide of Sequence V is blocked by staurosporine and sphingosine.

Biograph computer software was utilized to generate all computer-generated graphic models included in the Figures.

Identification of the Insulin Receptor Binding Domain

The insulin molecule B-chain residues B23-26 Gly-Phe-Phe-Tyr are involved directly in a receptor binding domain. It is also known that the C-terminal portions of the monomeric insulin B-chains bind inter se to form the insulin dimer. From a study of the dimer interface (see Figure 1A), it may be deduced that a receptor sequence for interaction in a similar fashion with the same C-terminal portion of the insulin monomer

(i) would contain two or three Phe or Tyr chains;

(ii) may have some homology with the C-terminal end of the insulin B-chain;

(iii) would be in a hydrophobic environment; and

(iv) would be in a β-sheet secondary structure.

Inspection of the known sequence of the receptor α subunit reveals various stretches containing at least two or three Phe or Tyr chains in close proximity. Of these, the 88-91 (Phe-Phe-Asn-Tyr) stretch was selected because:

(i) As Figure 2 shows, the sequence 84 to 91 has 5 residues homologous to the invariant or mostly invariant insulin residues 20-26.

(ii) As Figure 3 shows, a computer-generated secondary structure and hydropathy profile of the α subunit indicates that the receptor segment 78-94 containing the Phe-Phe-Asn-Tyr sequence is a β-sheet followed by a helical portion 95 to 103 and that the whole sequence is largely hydrophobic.

A synthetic 18 amino acid peptide corresponding to the receptor sequence 86-103 was synthesized on a solid support utilizing the p-alkoxybenzyl ester anchoring linkage of Wang, S.S., J.Am.Chem.Soc. 95:1328-1333 (1973). The amino acids were amino-protected by the N-fluorneylmethoxy-carbonylamino (Fmoc) group. The side chains were protected with t-butyl or other appropriate protecting groups. After synthesis the peptide was cleaved from the solid support by trifluoroacetic acid containing suitable solvents and other reagents to protect the peptide during this cleavage. The peptide was then passed through a Sephadex-G-10 and final purification is achieved by using a C-18 column

on an HPLC system. The exact molecular weight of the peptide was verified on a high resolution mass spectrometer. It was found to be highly hydrophobic as evidenced by substantial insolubility in less than 90% dimethylsulfoxide (DMSO). In water at high concentrations, the peptide formed a gel-like suspension and eventually precipitated as transparent crystalline-appearing structures.

The amino acid residues in this peptide are in the sequence depicted by the following Sequence I:

ARG-LEU-PHE-PHE-ASN-TYR-ALA-LEU-VAL-ILE-PHE-GLU-MET-
86 87 88 89 90 91 92 93 94 95 96 97 98

VAL-HIS-LEU-LYS-GLU
99 100 101 102 103

To test for insulin binding, a water suspension of the peptide was incubated at room temperature with a tracer of ^{125}I-insulin followed by simple centrifugation. The peptide was found to bind up to 30% of the tracer. The binding was displacable by an excess of unlabelled insulin. See Figure 6. The apparent dissociation constant was ~6 x 10^{-7}M. Non-specific binding was negligible. The peptide also bound ^{125}I-IGF I, but less well, while ^{125}I-EGF (epidermal growth factor) and ^{125}I-hGH (human growth hormone) showed no specific binding. These data correspond well to the predictions from sequence homologies in Figure 7.

This invention includes modifications of Sequence I in which additional residues corresponding to preceding and succeeding portions of the receptor α subunit are present. For example, the invention includes a Sequence II which also includes residues 83 to 85 of the insulin receptor α subunit as follows:

ARG-GLY-SER-ARG-LEU-PHE-PHE-ASN-TYR-ALA-LEU-VAL-ILE-
83 84 85 86 87 88 89 90 91 92 93 94 95

PHE-GLU-MET-VAL-HIS-LEU-LYS-GLU
96 97 98 99 100 101 102 103

The invention also includes modification of Sequence I in which one or more additional residues are added at one or both ends of the sequence to increase solubility. Specifically, the invention contemplates a Sequence III which includes

$(LYS)_x$-ARG-LEU-PHE-PHE-ASN-TYR-ALA-LEU-VAL-ILE-PHE-GLU-
86 87 88 89 90 91 92 93 94 95 96 97

MET-VAL-HIS-LEU-LYS-GLU-$(LYS)_y$
98 99 100 101 102 103

in which x and y are each 0, 1 or 2 with the provision that at least x or y is 1.

An additional Sequence IV has been synthesized for like solubility reasons:

LYS-ARG-GLY-SER-ARG-LEU-PHE-PHE-ASN-TYR-ALA-LEU-
83 84 85 86 87 88 89 90 91 92 93

VAL-ILE-PHE-GLU-MET-VAL-HIS-LEU-LYS-LYS
94 95 96 97 98 99 100 101 102

As appears from inspection, Sequence IV was produced by including residues 83-85, adding one LYS at the N-terminal, and replacing the negatively charged GLU at 103 by LYS, thus producing two positively-charged residues, LYS-ARG and LYS-LYS, at the N and C terminals, respectively.

Sequences V, VI and VII are modifications of Sequence IV achieved by replacing PHE-PHE at 88-89 with LEU-PHE (Sequence V), PHE-LEU (Sequence VI) and LEU-LEU (Sequence VII).

The invention further includes Sequences I to VII coupled to beads of polystyrene or other solid supports for use in solid phase assays and to keyhole limpet hemocyanin for use in the production of sequence antibodies. Included as well is the synthesis of an oligonucleotide corresponding to the Sequence I or II peptides and their expression in E. coli as part of a fusion protein with, for example, β-galactosidase or dihydrofolate reductase. Fusion may be to a synthetic IgG-binding domain from staphylococcus aureus protein A (see Lowenalder, B., et al. Gene 58:87-97 (1987)) to generate sequence antibodies for use in the performance of physical studies such as x-ray crystallography.

Figure 1B illustrates replacement of the side chains of residues CYS^{B19}, GLU^{B21}, GLY^{B23} and B26-30 of one of the insulin monomers shown in Figure 1A by the side chains of the residues occupying homologous positions in the insulin receptor sequence--specifically by ARG 83, SER 85, LEU 87 and ASN-TYR-ALA-LEU-VAL. More specifically, Figure 1B shows a molecular graphic model of the insulin sequence B19-B30 of one insulin monomer in the dimer interface replaced by sequence 83-94 of the insulin receptor. The similarity to the insulin dimer interface, Figure 1A, is striking.

This data indicates that residues 83 to 94 of the insulin receptor α subunit includes at least an effective portion of a domain involved in binding the active site of the insulin molecule.

Identification of the IGF-I Receptor Binding Domain

The involvement of Sequence VII of the IGF-I receptor residues 77-97 homologous to insulin receptor sequence 83-103 in IGF-I binding is evidenced by the following:

(i) Insulin and IGF-I bind to each other's receptors.

(ii) Sequence B23-26 of the insulin molecule is conserved in IGF-I molecule (GLY-PHE-TYR-PHE instead of GLY-PHE-PHE-TYR).

(iii) Sequence 83-97 of insulin receptor is highly conserved in IGF-I receptor.

(iv) Synthetic peptide (Sequence I) binds IGF-I.

(v) Figure 8C, a graphic representation, illustrates replacement of the side chains of residues CYS^{B19}, GLU^{B21}, GLY^{B23} and B^{26-30} of one of the insulin monomers shown in Figure 1A by the side chains of the residues occupying homologous positions in the IGF-I receptor sequence--specifically by ARG, TRP, LYS, LEU and ASN-TYR-ALA-LEU-VAL, and also shows striking similarity to the insulin dimer interface.

Accordingly, the invention also includes the following Sequence VIII of the IGF-I receptor:

ARG-GLY-TRP-LYS-LEU-PHE-TYR-ASN-TYR-ALA-LEU-VAL-ILE-
77 78 79 80 81 82 83 84 85 86 87 88 89

PHE-GLU-MET-THR-ASN-LEU-LYS-ASP
90 91 92 93 94 95 96 97

The invention further comprises physical and graphic representations of Sequence VIII and the use thereof in the design of drugs.

Demonstration of Insulinomimetic Properties

Insulinomimetic properties of the peptides of this invention is demonstrated by:

1. The ability, as compared to insulin, of these peptides to stimulate the incorporation of 3-[^{3}H] glucose into the lipids of isolated rat adipocytes.

2. A demonstration that insulin octapeptide B23-30 is totally inactive in the same lipogenesis assay (Figure 1).

3. A demonstration that the lipogenetic activity of the peptides is inactivated by polyclonal insulin antibody suggesting structurally similar peptide and insulin epitopes (Figure 3).

4. The lipogenetic activity of the peptides, like that of insulin, is inhibited by kinase C inhibitors sphingosine and staurosporine suggesting similar pathways of action (Figure 4).

5. A peptide with insulin receptor sequence 81-91 is totally inactive suggesting that the region 92-103 is important for observation of the insulinomimetic effect. Further, an antibody against peptide sequence 81-91 does not block the lipogenetic effect of Sequence V.

Example I

Comparative Lipogenesis Assay

The ability of Sequences IV through VII to stimulate the incorporation of 3-[^{3}H] glucose into lipids of isolated rat adipocytes was compared to insulin's.

The lipogenesis assay was conducted according to Smal, et al., J.Biol.Chem. 262:11071-11079 (1987). Dissected epididymal and retroperitoneal fat pads were digested under vigorous shaking at 37°C for 30 min with collagenase (1.0 mg/ml) in Krebs-Ringer-Heps (KRH) buffer, pH 7.4, 35 mg/ml dialyzed bovine serum

albumin (BSA), 0.27 mM glucose. After filtration on cheesecloth and 4 washes in KRH with 10 mg/ml BSA, the adipocytes were preincubated for 4 hours at 37°C in the same buffer.

The lipogenesis assay was performed in triplicate in 6 ml polyethylene vials by adding successively 400 µl of adipocyte suspension (80 X 10^3 cells/ml), 50 µl of KRH buffer pH 7.4 (1% BSA, 0.27 mM glucose) without hormone (basal lipogenesis) or with insulin or receptor-derived peptides at the indicated concentrations, and 50 µl D-[3-^{3}H]-glucose in a total volume of 0.5 ml. The vials were incubated 2 hours at 37°C under gentle shaking. The incubation was interrupted by adding 5 ml/tube of toluene scintillator (1 liter toluene + 0.3 g of 1,4bis[2-(4-methyl-5 phenyloxazolyl)] benzene and 5 g of 2,5-diphenyloxazole under vigorous shaking (30s to break the cells) followed by a rest of at least 1 hour to allow extraction of lipids into the toluene phase before counting. The counting efficiencies for the different samples were measured by internal standardization with quenched tritiated standards. The incorporation of D-[3-^{3}H]-glucose into lipids is expressed in cpm X 10^{-3}/tube $\pm$ 1 standard deviation.

As shown in Figure 10, all four peptides stimulated lipogenesis to an extent close to insulin's effect; the relative potency of the four peptides varied to some extent from experiment to experiment perhaps due to a somewhat limited solubility. The concentration of peptides required was much higher than that of insulin (note the two different horizontal scales).

Example II

Peptide Binding Concentration Range

The range of concentration over which a peptide of Sequence I binds to insulin is shown by Figure 11 to be similar to the range of concentration over which peptides IV through VII are shown to be active by Figure 10.

To provide the data illustrated by Figure 11, ^{125}I-insulin (1 X 10^{-11} M) was incubated overnight at room temperature in the absence or presence of 10 μg/ml unlabeled insulin with the indicated concentrations of peptide in assay buffer (100 mM Hepes, 120 mM NaCl, 5 mM KCL, 1.2 mM $MgSO_4$, 1 mM EDTA, 10 mM glucose, 15 mM $NaC_2H_3O_2$, 1% bovine serum albumin, pH 7.6). Bound and free tracer were separated by centrifuging the peptide suspension at 10,000 rpm for 10 min in a Beckman microfuge. The pellets were counted in a γ-counter.

Example III

Inhibition of Peptide Lipogenesis Activity by Anti-Insulin Antibody

Figure 12 shows that the lipogenetic activity of a Sequence V peptide is inactivated by the polyclonal anti-insulin antibody Sigma #1-8510.

To provide the data illustrated by Figure 12, a lipogenesis assay in the absence of hormone (basal), or with 10 μg/ml of insulin or 10 μg/ml of peptide V, in the absence or presence of anti-insulin antibody at a dilution of 1:500, was performed as described in detail with respect to Example I.

Example IV

Inhibition of Peptide Lipogenetic Activity by Kinase C Inhibitors

Co-pending application Serial No. 216,379 filed July 8, 1988 is entirely incorporated herein by reference. That application teaches that the stimulation of fat cell lipogenesis by insulin is blocked reversibly by the action of a kinase C inhibitor such as kinase C or staurosporine.

Figure 13 illustrates a like result with the peptide of Sequence V.

To generate the data depicted by Figure 13, a lipogenesis assay in the absence of hormone (basal), or with 10 μg/ml of insulin or 100 μg/ml of peptide V, in the absence or presence of kinase inhibitors staurosporine (10 μg/ml) or sphingosine (100 μM), was performed as described in detail with respect to Example I.

Example V

Activity of Insulin Receptor Sequence 92-103

This example indicates insulinomimetic activity of the insulin receptor sequence 92-103 (Sequence IX):

ALA-LEU-VAL-ILE-PHE-GLU-MET-VAL-HIS-LEU-LYS-GLU

92 93 94 95 96 97 98 99 100 101 102 103

The first aspect of this example is that the insulin receptor sequence 81 to 91 is totally inactive lipogenetically. Specifically, when peptide with sequence 81 to 91 is used instead of peptides IV through VII in the experiment described in Example I, lipogenesis is not stimulated above basal.

A second aspect of the example is that an antibody against peptide 81-91 is unable to block the effect of the peptide of Sequence V.

A further specific embodiment of this invention includes synthetic and purified peptide sequences corresponding to Sequence IX, insulinomimetic drugs containing such peptides, and the treatment of diabetic and related diseases by the administration of such drugs.

I CLAIM:

1. A purified natural or a synthetic peptide which consists essentially of a portion of Sequence I or Sequence II or Sequence III which binds the human insulin molecule.

2. A purified natural amino acid residue sequence comprising Sequence I.

3. A purified natural amino acid residue sequence comprising Sequence II.

4. A synthetic peptide which consists essentially of amino acid residue Sequence I, or amino acid residue Sequence II, or amino acid residue Sequence III.

5. A purified or a synthetic fragment of the human insulin receptor α subunit which comprises at least so much of Sequence I as is effective to bind the human insulin molecule, said fragment having a ternary structure as depicted by any of Figures 4A, 4B, 4C, 4D, 5A or 5B.

6. A three dimensional molecular model including residues 83-94 of the insulin receptor α subunit as depicted by any of Figures 4A, 4B, 4C or 4D.

7. A three dimensional molecular model comprising at least so much of the graphic model depicted by Figures 4A, 4B, 4C, 4D, 5A or 5B as is effective to bind the human insulin molecule.

8. A graphical representation of at least so much of residues 83-94 of the insulin receptor α subunit as is effective to bind the human insulin molecule.

9. A computer generated graphic representation as defined by claim 8.

10. The computer generated graphic representations depicted by Figures 4A, 4B, 4C or 4D.

11. The method of designing an insulinomimetic drug which comprises using as a template a graphic representation of at least so much of residues 83-94 of the insulin receptor α subunit as is effective to bind the human insulin molecule.

12. The method as defined by claim 11 in which the graphic representation is computer generated.

13. The method as defined by claim 11 in which a graphic representation as depicted by Figures 4A, 4B, 4C or 4D is used.

14. A purified natural or synthetic peptide which consists essentially of a portion of Sequence IV which binds the insulin-like growth factor I molecule.

15. A purified natural amino acid sequence comprising Sequence IV.

16. A purified or synthetic fragment of the insulin-like growth factor I receptor which comprises at least so much of Sequence IV as is effective to bind the IGF-I molecule, said fragment having a ternary structure as depicted by Figures 9A, 9B, 9C and 9D.

17. A three dimensional molecular model including residues 77-97 of the IGF-I receptor as depicted by any of Figures 9A, 9B, 9C or 9D.

18. A three dimensional molecular model comprising at least so much of the graphic model depicted by Figures 9A, 9B, 9C or 9D as is effective to bind the IGF-I molecule.

19. A graphic representation of at least so much of residues 77-97 of the IGF-I receptor as is effective to bind the human insulin molecule.

20. A computer generated graphic representation as defined by claim 19.

21. The computer generated graphic representation depicted by Figures 9A, 9B, 9C and 9D.

22. The method of designing a drug which comprises using as a template a graphic representation of at least so much of the residues 77-97 of the IGF-I receptor as is effective to bind the IGF-I molecule.

23. The method as defined by claim 22 in which the graphic representation is computer generated.

24. The method as defined by claim 23 in which the graphic representation is depicted by Figures 9A, 9B, 9C or 9D is used.

25. An insulinomimetic drug comprising a synthetic or purified amino acid residue sequence corresponding to a portion of the human insulin binding site.

26. An insulinomimetic drug comprising a synthetic or purified amino acid residue sequence corresponding to any of Sequences I through IX.

27. An insulinomimetic drug comprising an amino acid residue sequence including the human insulin receptor binding site.

28. A purified or synthetic fragment of the human insulin α subunit, said fragment having a ternary structure as depicted by Figure 5A or 5B.

29. A three-dimensional molecular model comprising at least so much of the graphic model depicted by Figure 5A or 5B as is effective to bind the human insulin molecule.

30. An isolated fragment of the amino acid sequence of the human insulin receptor α subunit, said fragment including an insulin binding site or an insulin-like growth factor I binding site.

31. A non-naturally occurring peptide consisting essentially of an amino acid residue sequence selected from the group consisting of

ARG-GLY-SER-ARG-LEU-PHE-PHE-ASN-TYR-ALA-LEU-VAL-ILE-
 83 84 85 86 87 88 89 90 91 92 93 94 95
PHE-GLU-MET-VAL-HIS-LEU-LYS-GLU
 96 97 98 99 100 101 102 103

and

$(LYS)_x$-ARG-LEU-PHE-PHE-ASN-TYR-ALA-LEU-VAL-ILE-PHE-
 86 87 88 89 90 91 92 93 94 95 96

GLU-MET-VAL-HIS-LEU-LYS-GLU-$(LYS)_y$
 97 98 99 100 101 102 103

and

LYS-ARG-GLY-SER-ARG-LEU-PHE-PHE-ASN-TYR-ALA-LEU-VAL-
 83 84 85 86 87 88 89 90 91 92 93 94

ILE-PHE-GLU-MET-VAL-HIS-LEU-LYS-LYS
 95 96 97 98 99 100 101 102

and

LYS-ARG-GLY-SER-ARG-LEU-LEU-PHE-ASN-TYR-ALA-LEU-VAL-
 83 84 85 86 87 88 89 90 91 92 93 94

ILE-PHE-GLU-MET-VAL-HIS-LEU-LYS-LYS
 95 96 97 98 99 100 101 102

and

LYS-ARG-GLY-SER-ARG-LEU-PHE-LEU-ASN-TYR-ALA-LEU-VAL-
83 84 85 86 87 88 89 90 91 92 93 94

ILE-PHE-GLU-MET-VAL-HIS-LEU-LYS-LYS
95 96 97 98 99 100 101 102

and

LYS-ARG-GLY-SER-ARG-LEU-LEU-LEU-ASN-TYR-ALA-LEU-VAL-
83 84 85 86 87 88 89 90 91 92 93 94

ILE-PHE-GLU-MET-VAL-HIS-LEU-LYS-LYS
95 96 97 98 99 100 101 102

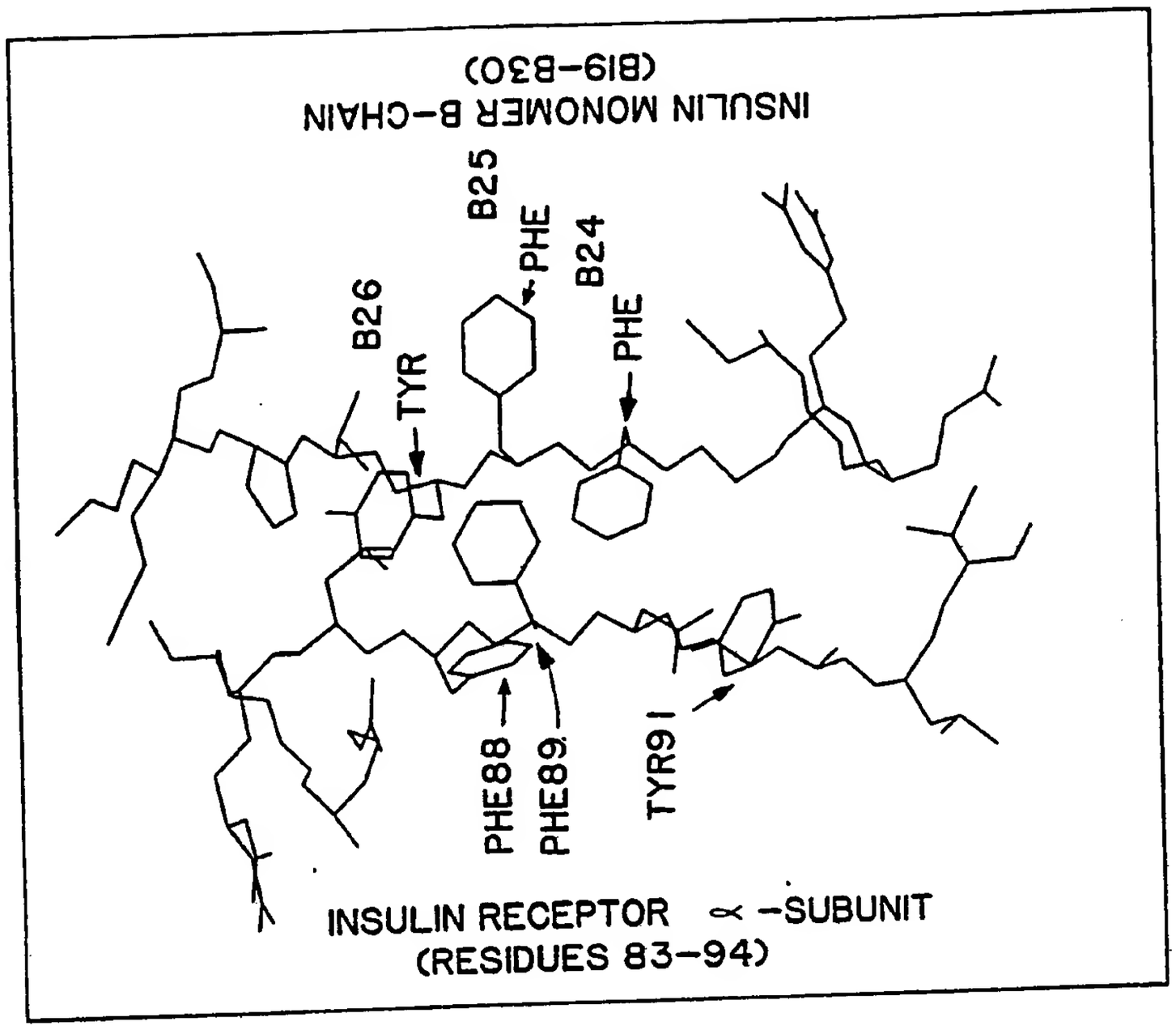

INSULIN MONOMER B-CHAIN
(B19-B30)
B25
PHE
B24
PHE
B26
TYR
PHE88
PHE89
TYR91
INSULIN RECEPTOR ∝-SUBUNIT
(RESIDUES 83-94)

FIG. 1B

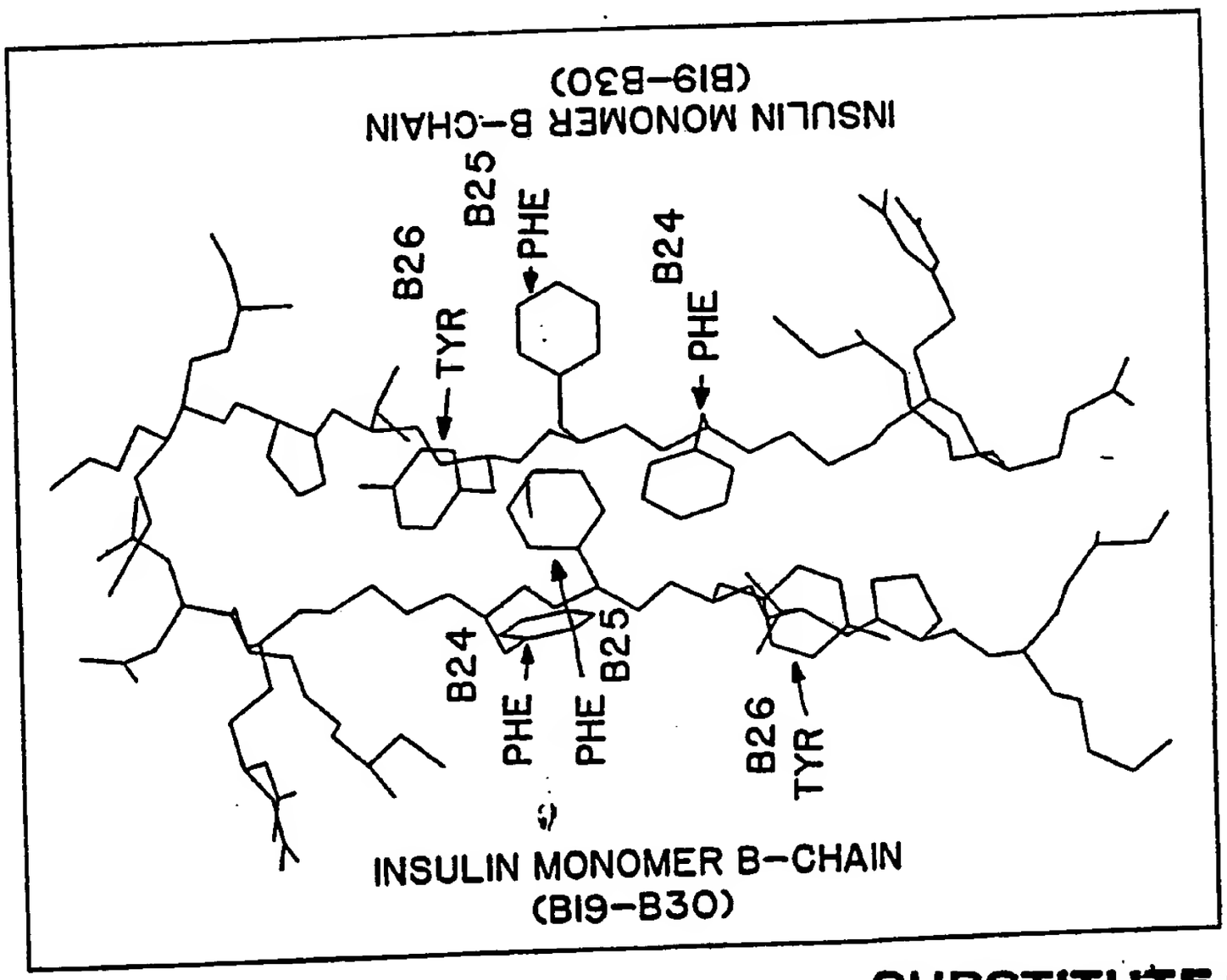

INSULIN MONOMER B-CHAIN
(B19-B30)
B25
PHE
B26
TYR
B24
PHE
B24
PHE
B25
PHE
B26
TYR
INSULIN MONOMER B-CHAIN
(B19-B30)

FIG. 1A

HOMOLOGY OF VARIOUS RECEPTOR FRAGMENTS CONTAINING
AT LEAST TWO AROMATIC SIDE-CHAINS WITH THE C-TERMINAL END OF INSULIN B-CHAIN

* Invariant or mostly invariant insulin residues

	B	19	20	21	22	23	24	25		26	27	28	29	30		
INSULIN		CYS	GLY	GLU	ARG	GLY	PHE	PHE	--	TYR	THR	PRO	LYS	THR		
RECEPTOR	83[1]	ARG	GLY	[SER]	ARG	[LEU]	PHE	PHE	ASN	TYR	[ALA]	LEU	VAL	[ILE]	PHE	GLU 98
	226	VAL	ALA	CYS	ARG	ASN	PHE	TYR	LEU	ASP	GLY	[ARG]	CYS	VAL	GLU	THR 241
	239	GLU	CYS	PRO	PRO	PRO	TYR	TYR	[HIS]	PHE	GLN	ASP	TRP	ARG	[CYS]	[VAL] 254
	376	[LEU]	VAL	[SER]	LEU	[SER]	PHE	PHE	ARG	LYS	LEU	[ARG]	LEU	[ILE]	ARG	GLY 390
	395	ILE	GLY	ASN	TYR	[SER]	PHE	TYR	ALA	LEU	ASP	ASN	GLN	ASN	LEU	ARG 410
	422	THR	GLN	GLY	LYS	[LEU]	PHE	PHE	[HIS]	TYR	ASN	[PRO]	LYS	LEU	[CYS]	LEU 436
	501	[LEU]	GLY	PHE	MET	[LEU]	PHE	TYR	LYS	GLU	[ALA]	[PRO]	TYR	GLN	ASN	[VAL] 515

First Residue Number (Ullrich's Numbering)

Last Residue Number

---

1 Synthetic peptide 86-103 binds insulin

FIG. 2

COMPUTER PREDICTION OF -SUBUNIT
SECONDARY STRUCTURE/HYDROPHOBICITY

FIG. 3-1

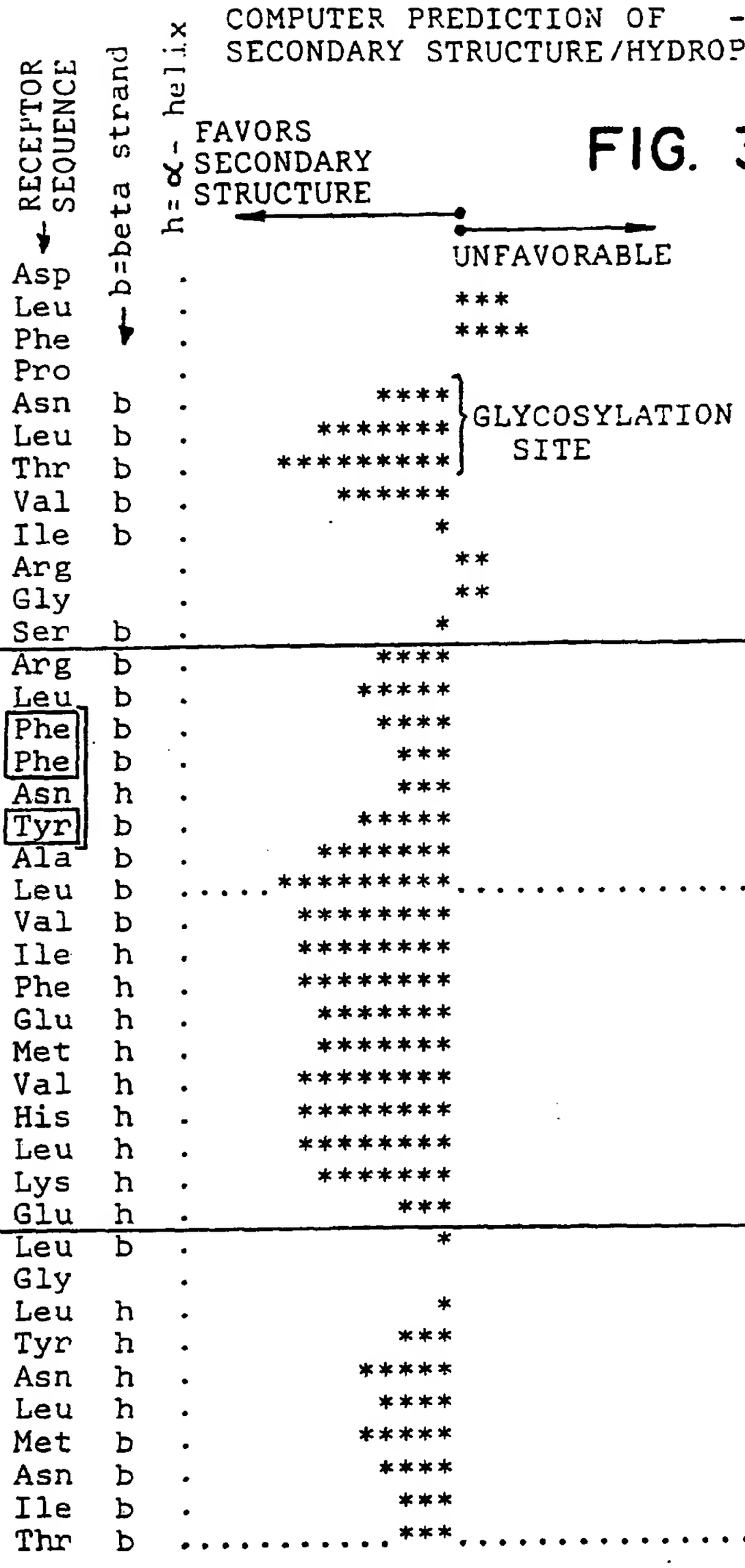
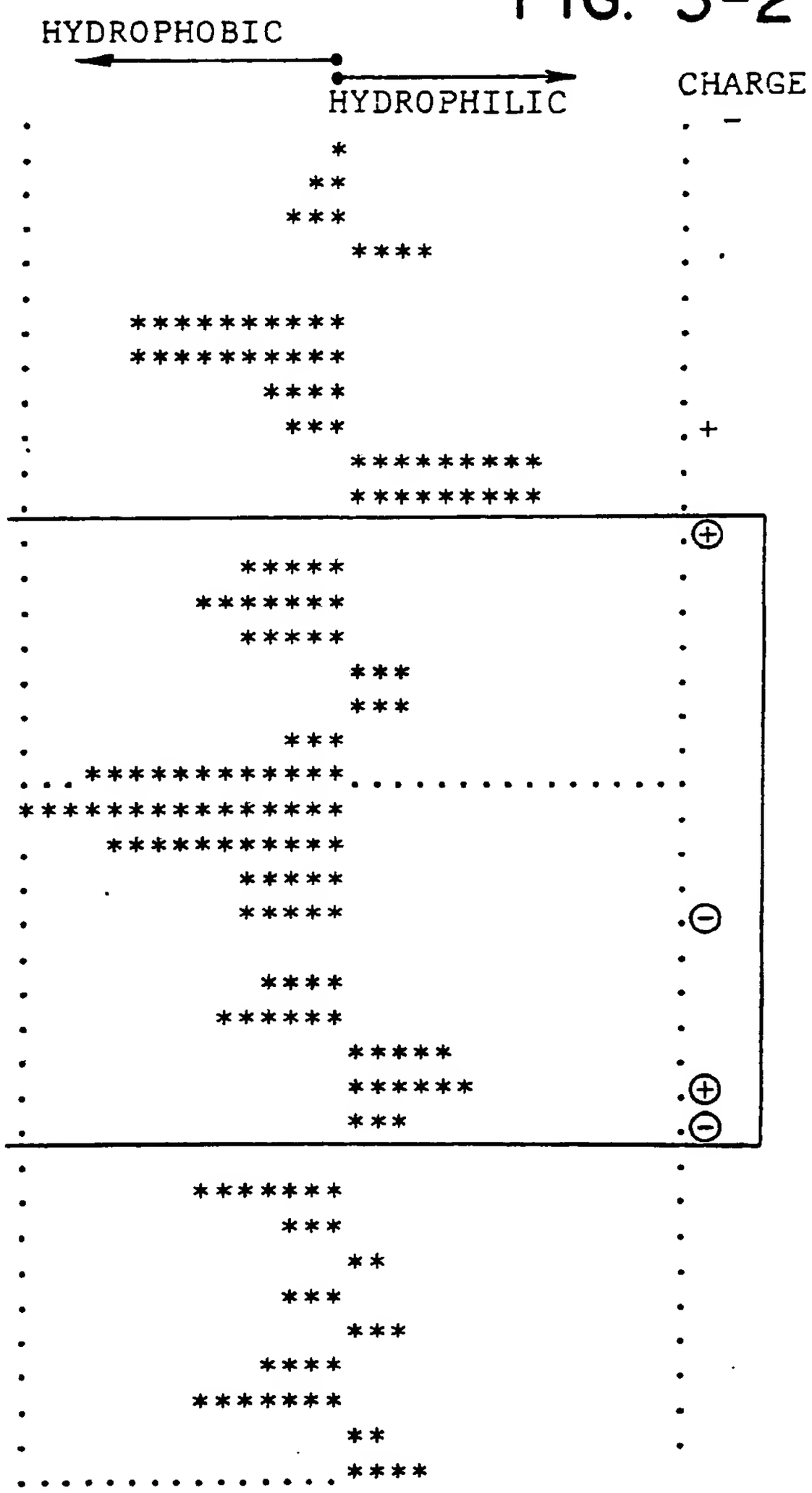


FIG. 3-2



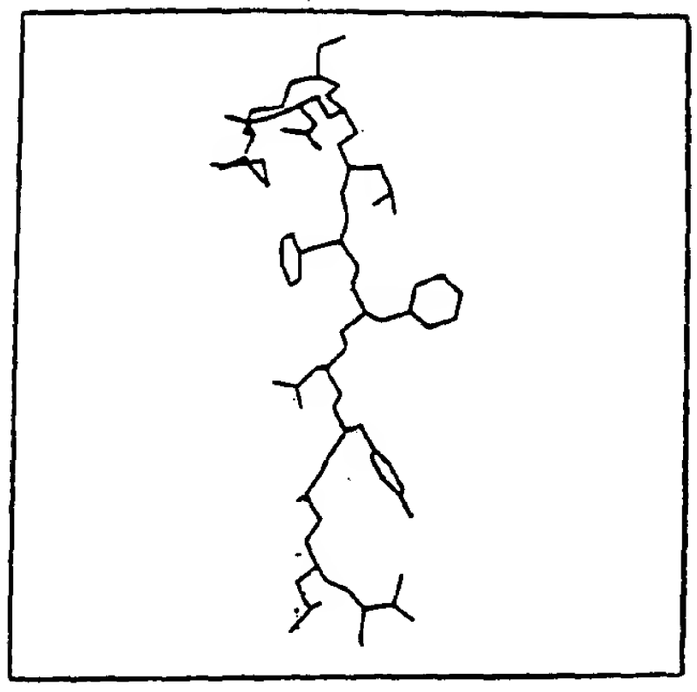


FIG. 4A

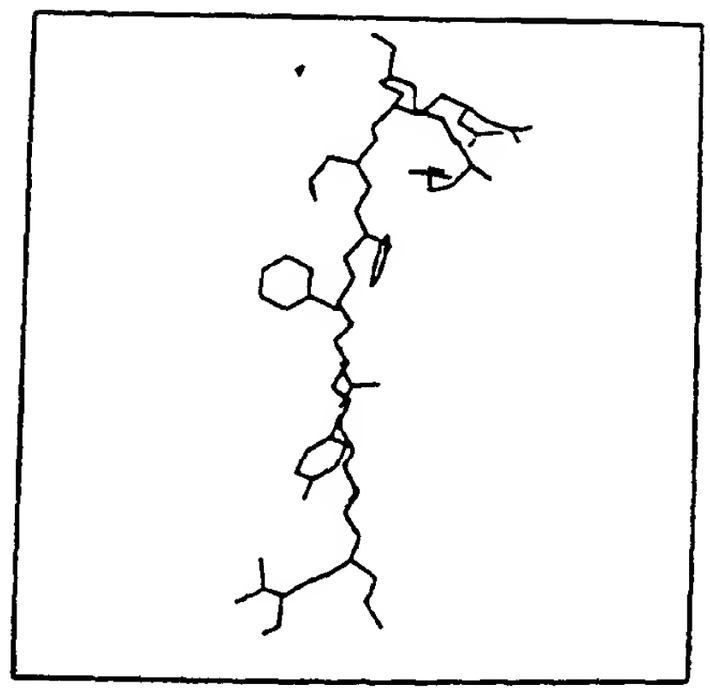


FIG. 4B

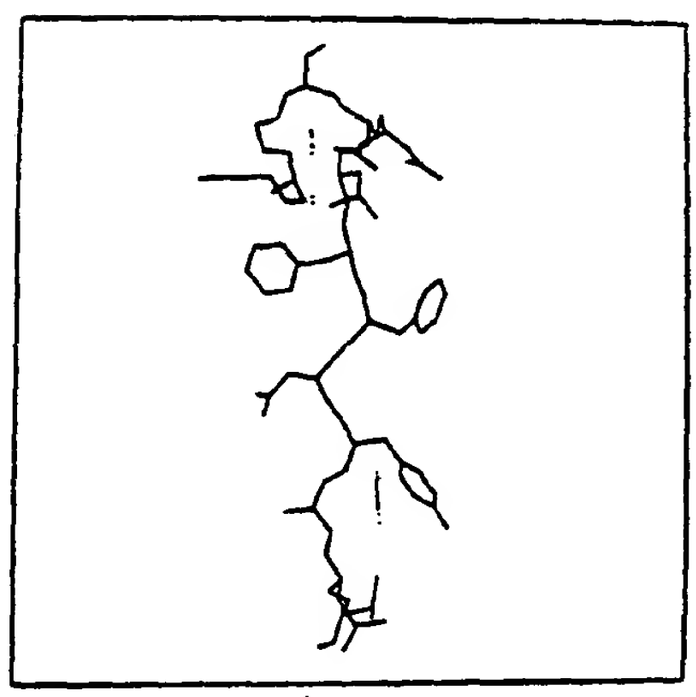


FIG. 4C

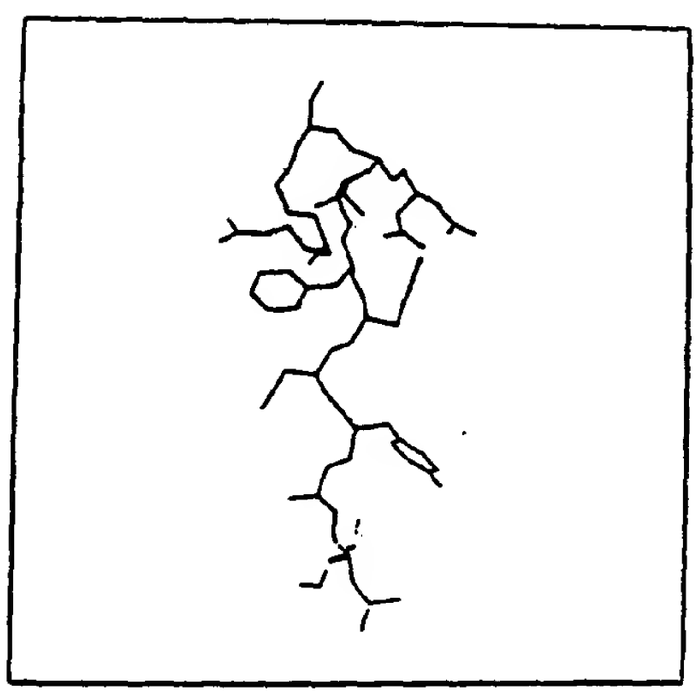


FIG. 4D

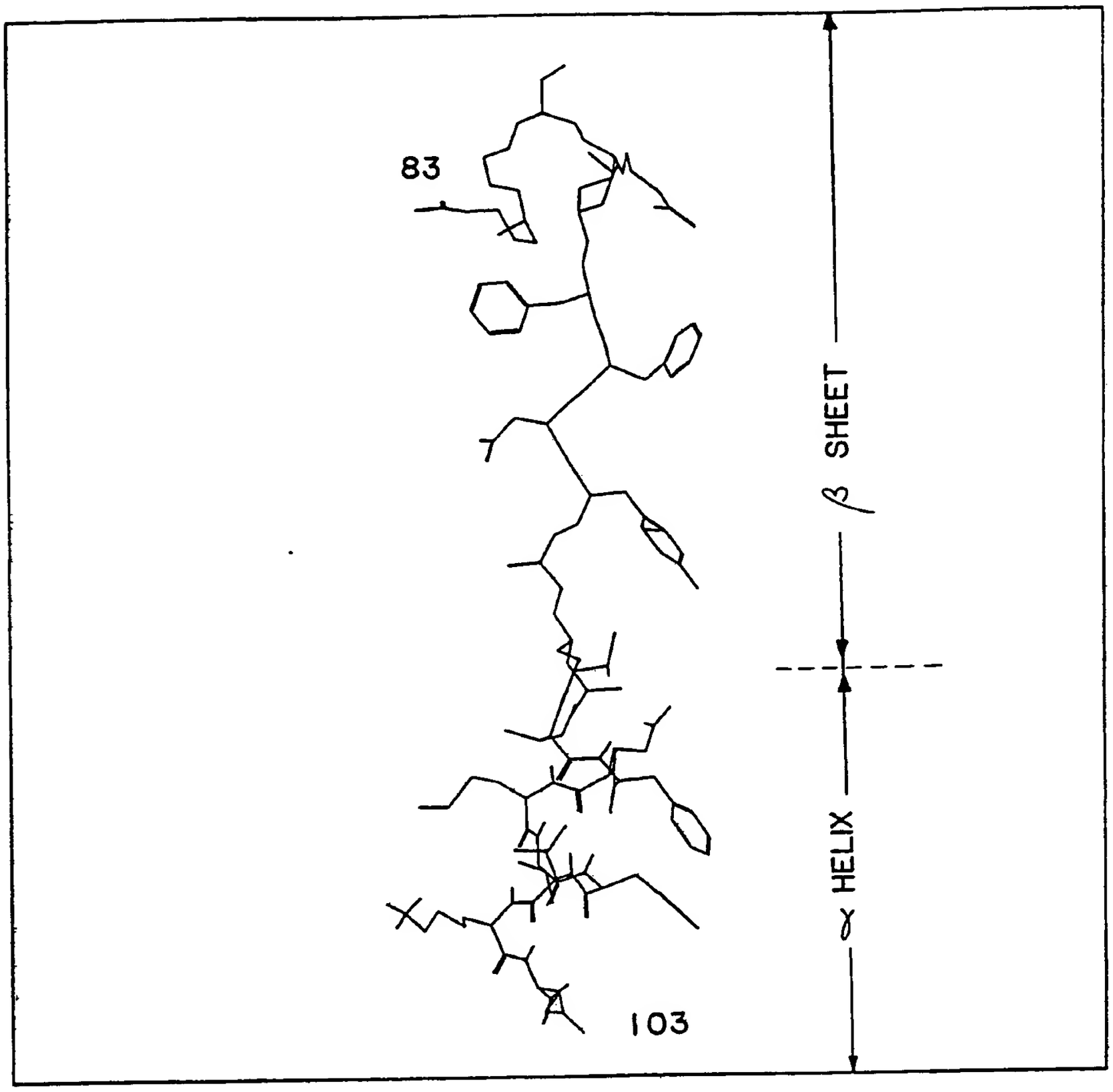


FIG. 5A

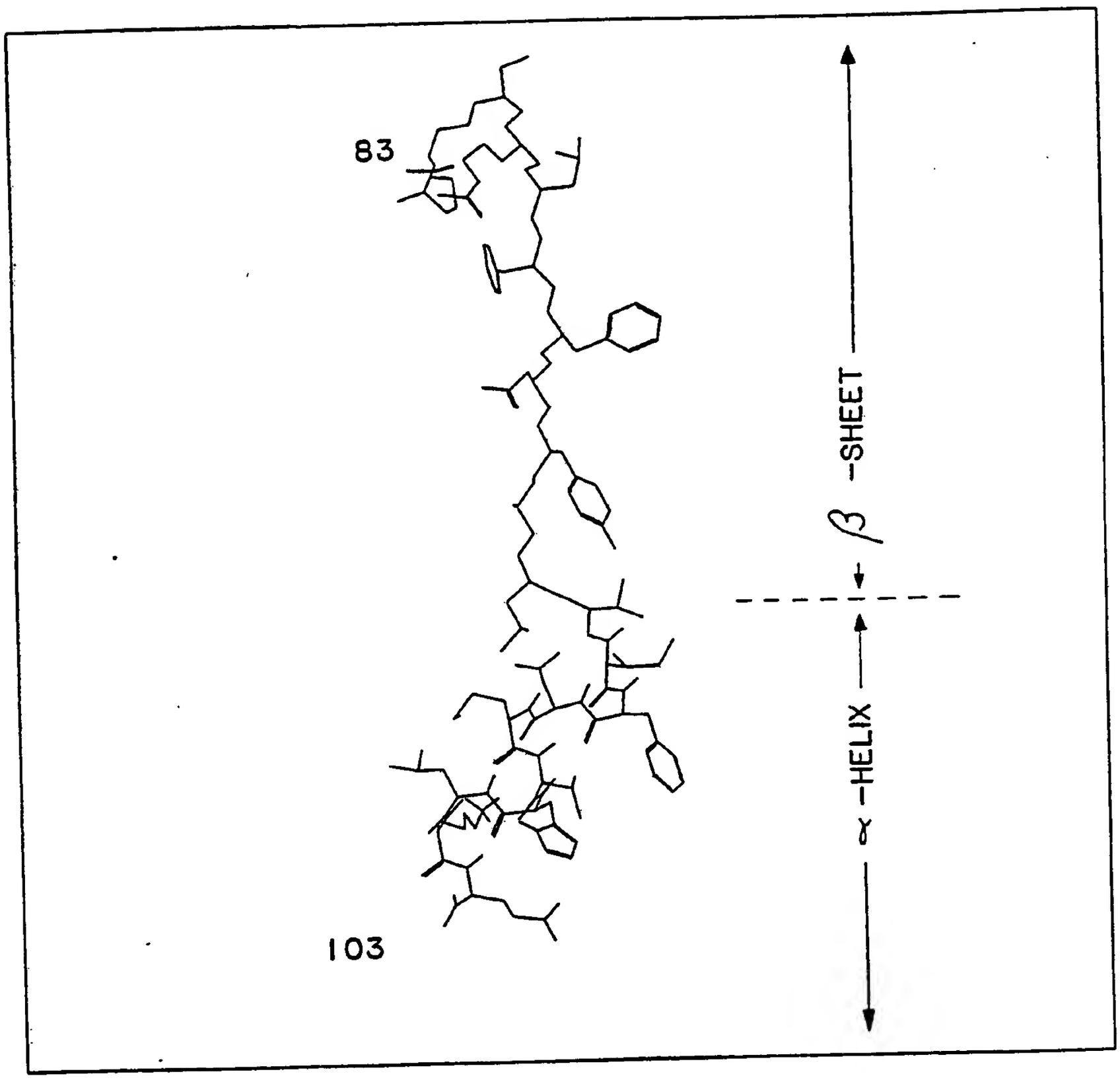
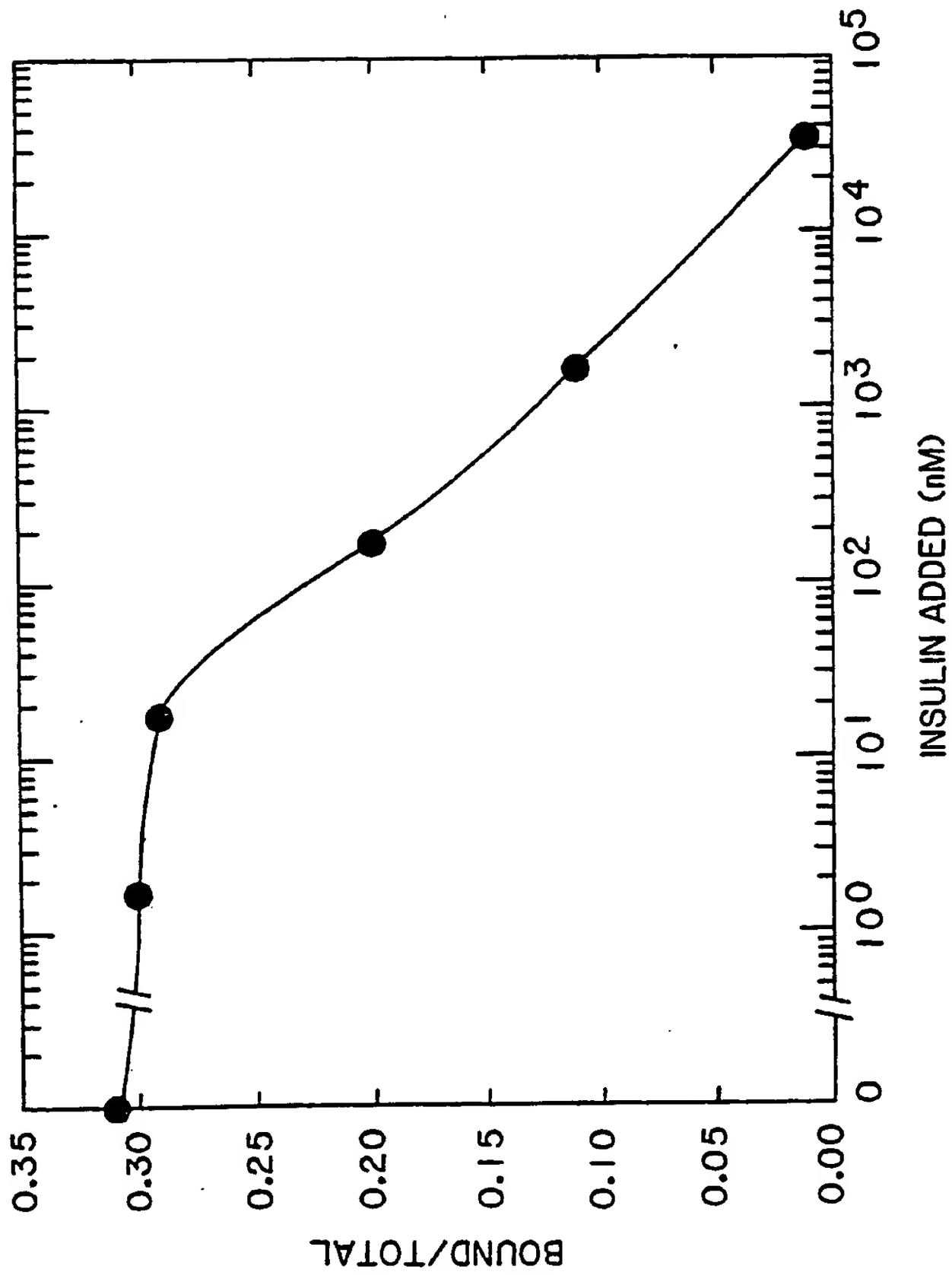


FIG. 5B

FIG. 6

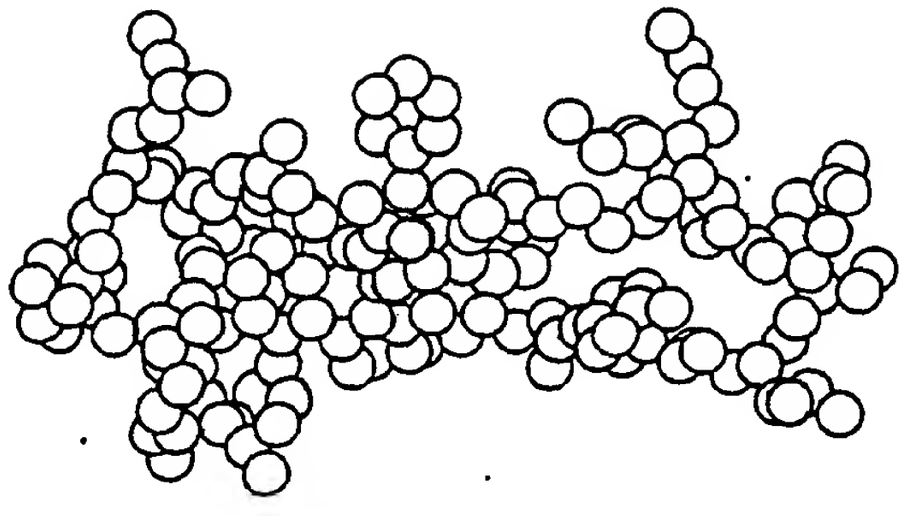

BOUND/TOTAL
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
INSULIN ADDED (nM)
0
10^0
10^1
10^2
10^3
10^4
10^5

HOMOLOGY BETWEEN C-TERMINAL ENDS OF INSULIN AND
IGF-I B-CHAINS, AND RECEPTORS FOR INSULIN, IGF I AND EGF

	B	19	20	21	22	23	24	25			26	27	28	29	30		
		COOPERATIVE SITE															
INSULIN		CYS	GLY	GLU	ARG	GLY	PHE	PHE	--	--	TYR	THR	PRO	LYS	THR		
INS. REC.		ARG	GLY	SER	ARG	LEU	PHE	PHE	ASN	--	TYR	ALA	LEU	VAL	ILE	PHE	GLU
IGF1 REC.		ARG	GLY	TRP	LYS	LEU	PHE	TYR	ASN	--	TYR	ALA	LEU	VAL	ILE	PHE	GLU
IGF1		CYS	GLY	ASP	ARG	GLY	PHE	TYR	--		PHE	ASN	--	LYS	PRO	THR	
EGF REC.		ARG	GLY	ASN	MET	TYR	TYR	GLU	ASN	SER	TYR	ALA	LEU	VAL	ILE		

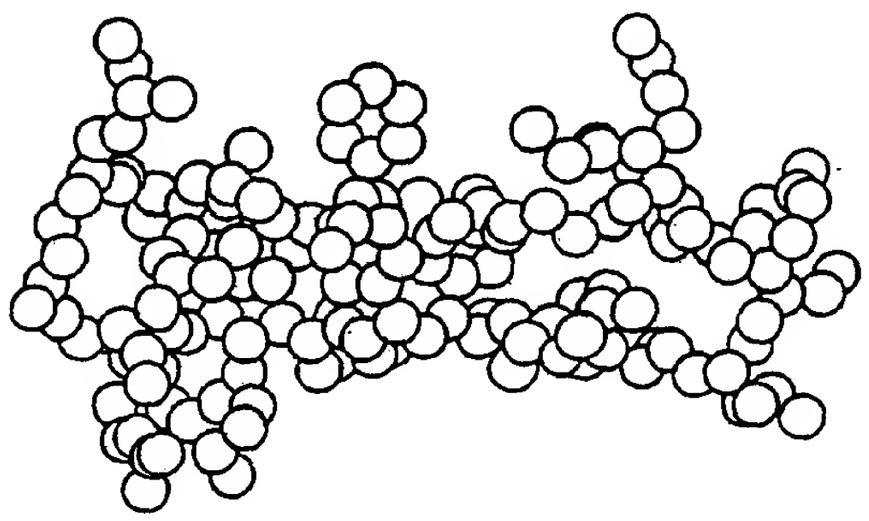
FIG. 7

FIG. 8C



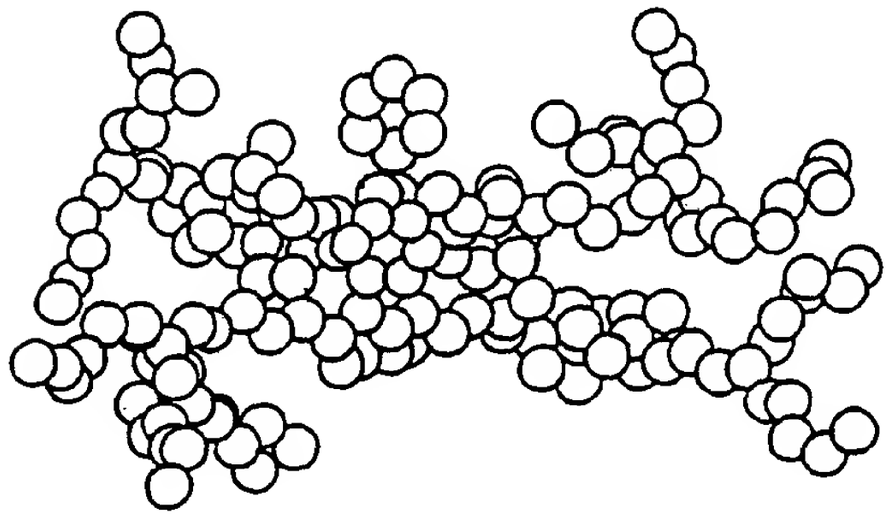
IGF I RECEPTOR DOMAIN–INSULIN PAIR

FIG. 8B



INSULIN RECEPTOR DOMAIN–INSULIN PAIR

FIG. 8A



INSULIN–INSULIN PAIR (DIMER)

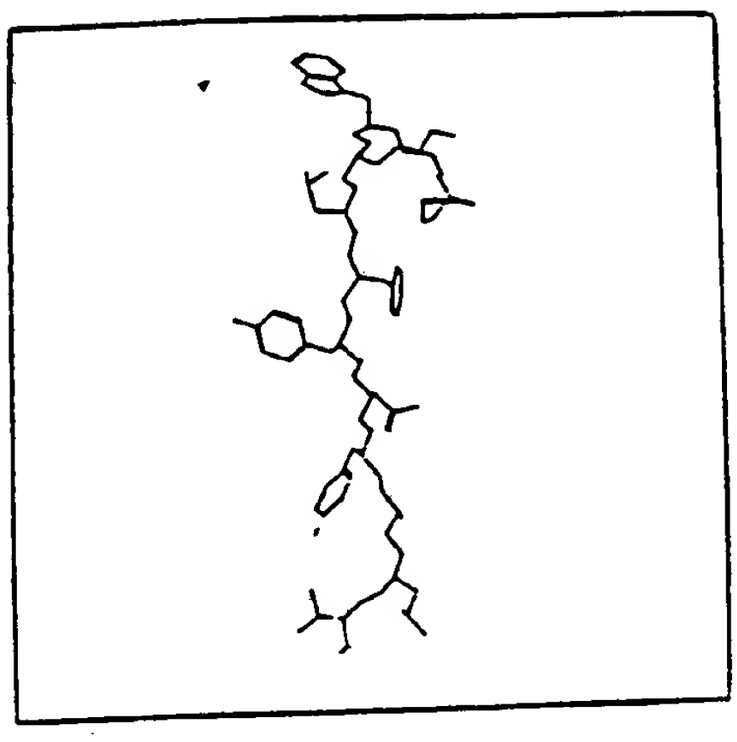


FIG. 9A

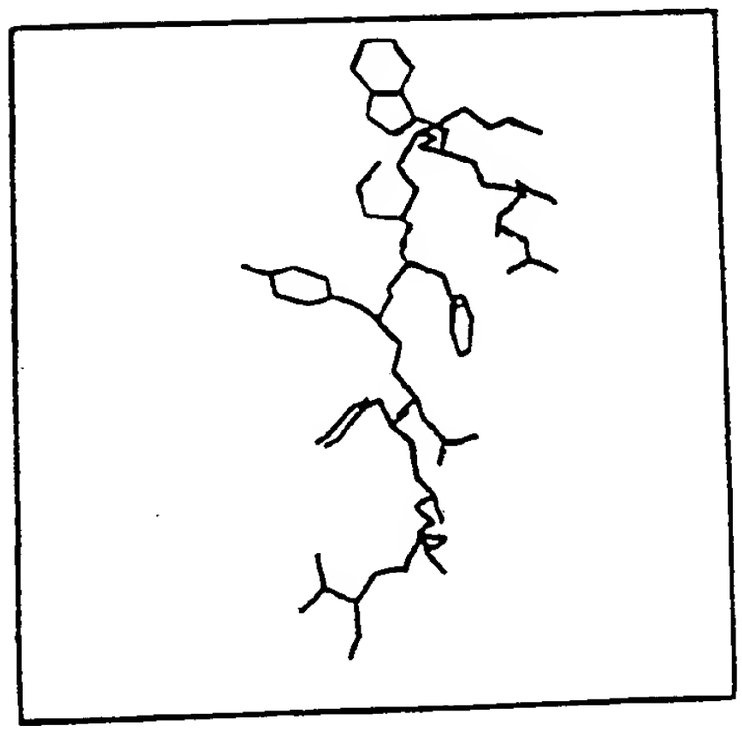


FIG. 9B

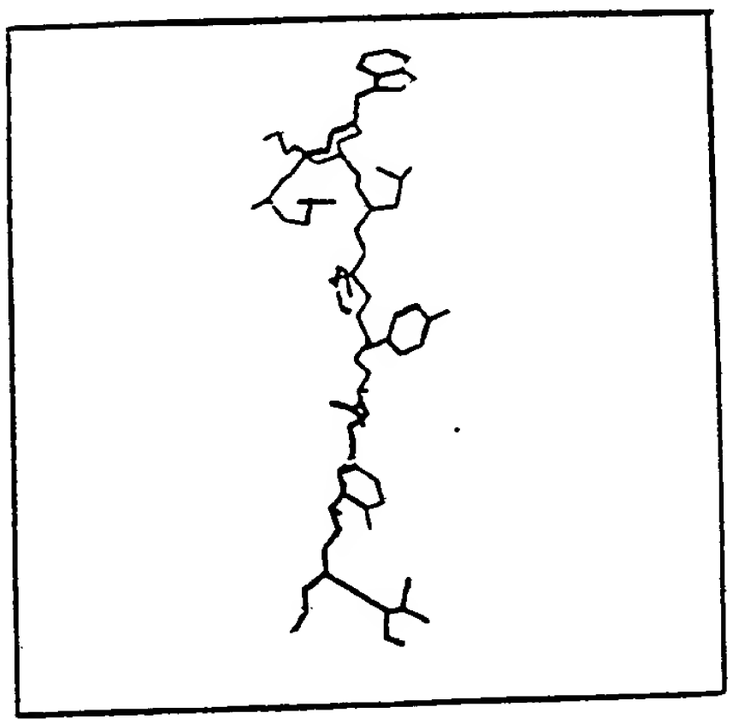


FIG. 9C

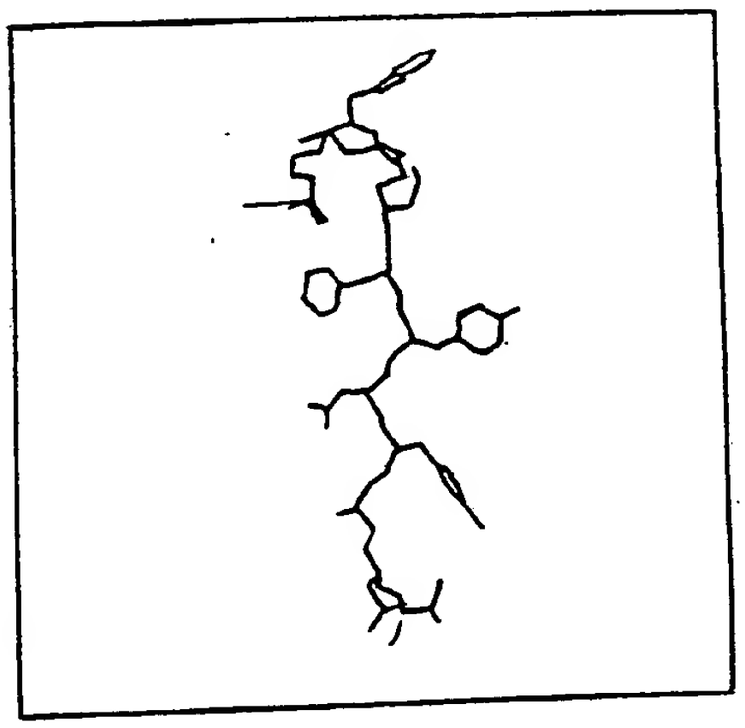


FIG. 9D

FIG. 10

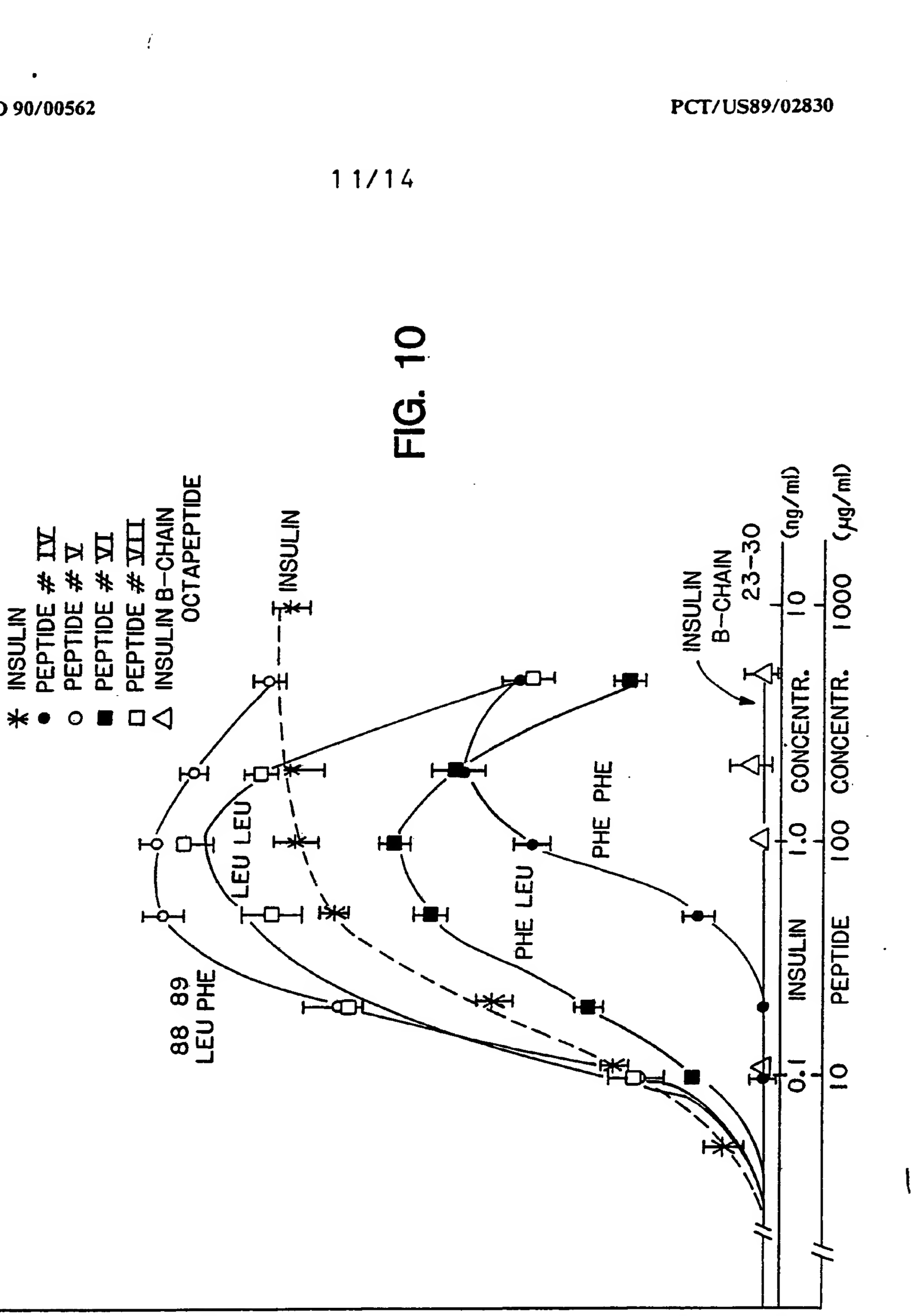
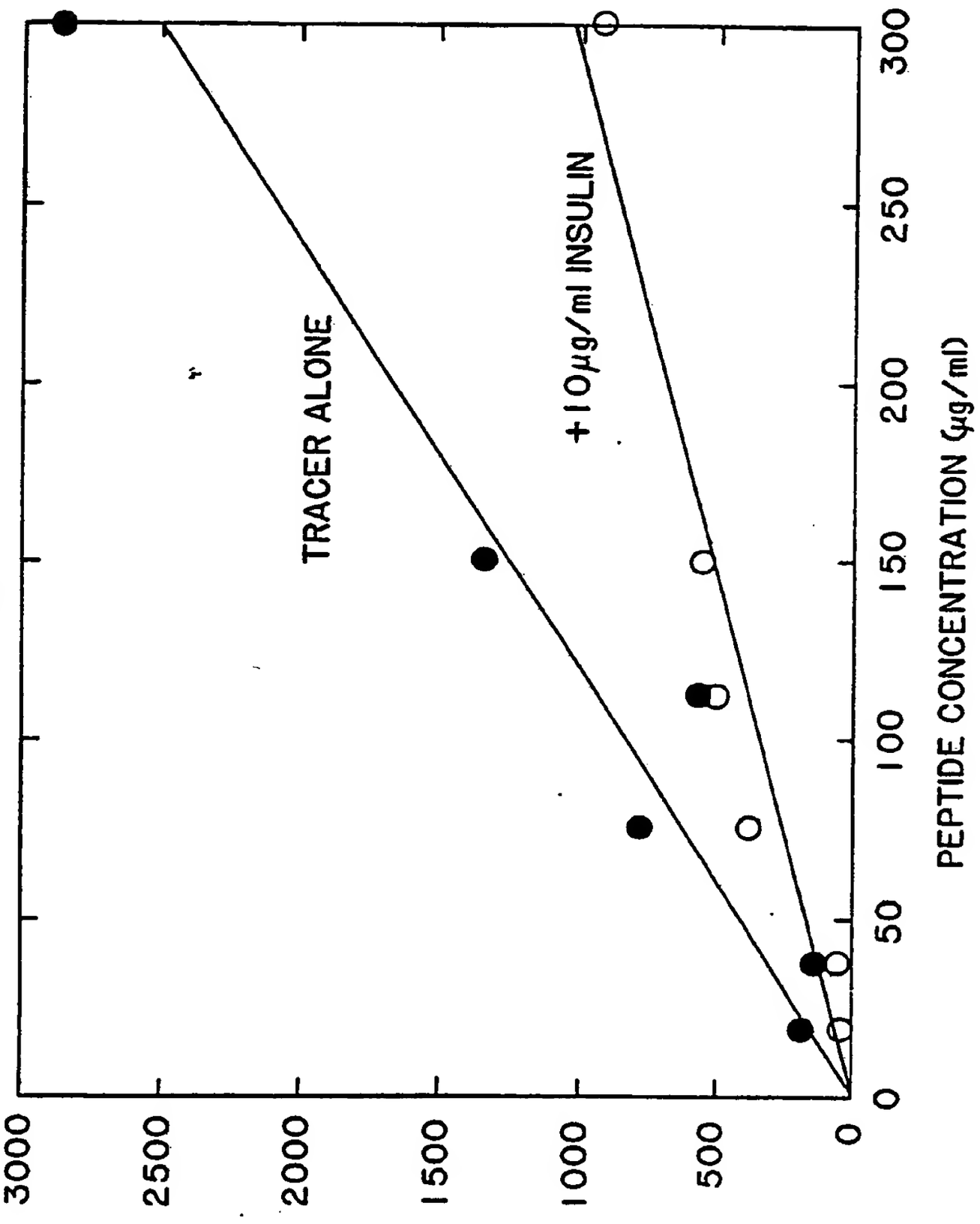

3H-GLUCOSE INCORPORATED INTO LIPIDS (CPM/TUBE X 10^{-3} ± 1 S.D.)
PEPTIDE CONCENTR. (μg/ml)
INSULIN CONCENTR. (ng/ml)
0.1
1.0
10
100
1000
INSULIN
PEPTIDE
INSULIN B-CHAIN 23-30
PHE PHE
PHE LEU
LEU LEU
88 89 LEU PHE
INSULIN
INSULIN B-CHAIN OCTAPEPTIDE
PEPTIDE # VIII
PEPTIDE # VI
PEPTIDE # V
PEPTIDE # IV
INSULIN

FIG. 11


Cpm BOUND
3000
2500
2000
1500
1000
500
0
TRACER ALONE
+10μg/ml INSULIN
0
50
100
150
200
250
300
PEPTIDE CONCENTRATION (μg/ml)

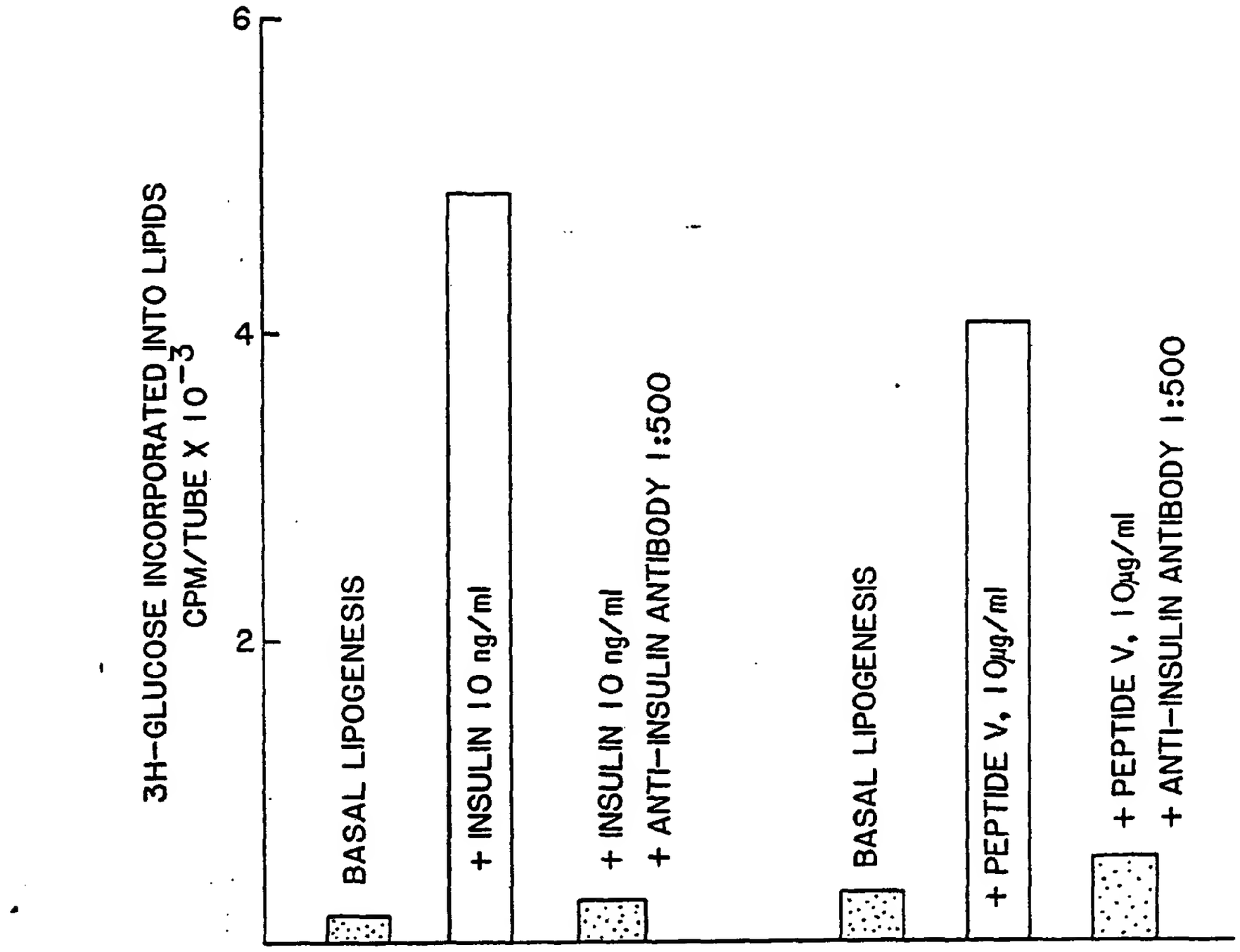

6
4
2
3H-GLUCOSE INCORPORATED INTO LIPIDS
CPM/TUBE X 10⁻³
BASAL LIPOGENESIS
+ INSULIN 10 ng/ml
+ INSULIN 10 ng/ml
+ ANTI-INSULIN ANTIBODY 1:500
BASAL LIPOGENESIS
+ PEPTIDE V, 10µg/ml
+ PEPTIDE V, 10µg/ml
+ ANTI-INSULIN ANTIBODY 1:500

FIG. 12

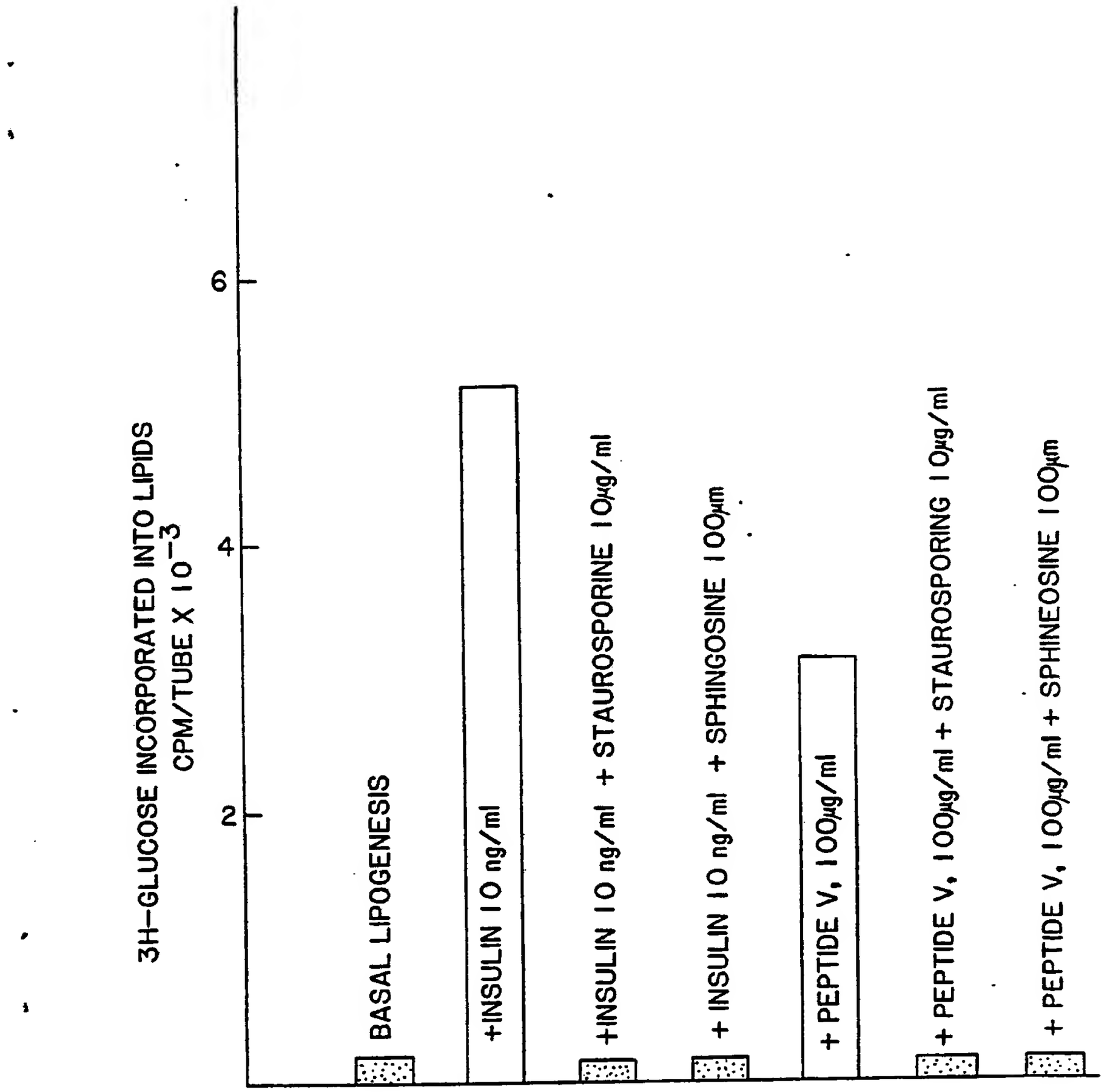

3H-GLUCOSE INCORPORATED INTO LIPIDS
CPM/TUBE X 10⁻³
2
4
6
BASAL LIPOGENESIS
+INSULIN 10 ng/ml
+INSULIN 10 ng/ml + STAUROSPORINE 10µg/ml
+ INSULIN 10 ng/ml + SPHINGOSINE 100µm
+ PEPTIDE V, 100µg/ml
+ PEPTIDE V, 100µg/ml + STAUROSPORING 10µg/ml
+ PEPTIDE V, 100µg/ml + SPHINEOSINE 100µm

FIG. 13

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02830

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 7/08
U.S.Cl.: 530/326

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S	530/326

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
	Cell, vol. 40, April 1985, Y. Ebina,	
A	"The human insulin receptor cDNA the structural basis for hormone-activated transmembrane signalling", pages 747-758, See entire document.	1,2,4
.Y	Nature, vol. 313, 28 February 1985, A. Ullrich, "Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes", pages 756-761, see page 760, col. 1, second paragraph	1,2,4

* Special categories of cited documents: [16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05 December 1989	03 JAN 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/USA	T. Wessendorf

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,Y	US,A 4,761,371 (BELL) 02 August 1988, see col. 18, Table 1, lines 5-24	1,2,4
A	The EMBO Journal, vol.5, no.10, 1986, A. Ullrich, "Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity", pages 2503-2512, See the entire document.	1,2,4
A	Chemical Abstracts, vol. 99, no.7, 1983 (Columbus, Ohio, USA), T. BLUNDELL, "Tertiary structures, receptor binding, and antigenicity of insulin-like growth factors", see page 64, col. 2, abstract no. 48026j, Fed. Proc. Fed. Am Soc. Exp. Biol. 1983, 42(9), 2592-7(Eng).	1,2,4
A	Chemical Abstracts, vol. 105, no. 23, 1986 (Columbus, Ohio, USA), F. Yamaguchi, "Comparison of insulin-like growth factor I receptor and insulin receptor purified from human placental membranes", see page 126, col. 1, abstract no. 203833h, J. Biol. Chem. 1986, 261(35) 16727-31 (Eng).	1,2,4

FURTHER INFORMATI N CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See Attached Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1,2,4

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to Form PCT/ISA/210 - Part VI

Group I -Peptides (Natural or Synthetic)-claims 1-4,

Subgroup I: Species of Sequence I (claims 1, 2 and 4)
Subgroup II: Species of Sequence II (claims 1, 3 and 4)
Subgroup III: Species of Sequence III (claims 1, and 4)

Group II -A synthetic peptide fragment of human insulin receptor -claim 5,

Group III -Peptides including residues 83 94-claims 6-13.

Group IV -Natural or synthetic peptide of Sequence IV which binds to insulin like growth factor I molecule-Claims 14 and 15.

Group V -A synthetic peptide fragment of IGF-I-claim 16.

Group VI - Peptides including residues 77-97 of the IGF-1 receptor -Claims 17-24

Group VII -An insulinomimetic drug of a synthetic or purified peptide corresponding to a portion of the human insulin binding site -Claim 25.

Group VIII -Insulinomimetic drug claim 26.

Subgroups: Species of I-IX

Group IX -Insulinomimetic drug comprising an amino acid residue sequence including the human insulin receptor binding site-claim 27.

Attachment to Form PCT/ISA/210 -Part VI

Group X -Synthetic fragment of the human insulin subunit-
claims 28-30.

Groups XI -Synthetic peptide
Claim 31

Subgroups I: Specie of residues 83-103
II: Specie of residues 85-104
III-VI: Species of residues 82-103

Each of these groups and subgroups contain peptides or amino acid residues or fragments that are patentably distinct. Each of these peptides differ structurally from one another. There is no common chemical core to suggest one peptide in the claim over the other.

Applicant is invited to identify the groups or subgroups (species) which applicant considers are obvious variants of one another and to which extent, examination would be further extended.